Antimicrobial Activity of Allocasuarina littoralis methanolic leaf extracts

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Citation

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Abstract

The antimicrobial activity of a methanolic extract of Allocasuarina littoralis leaves was investigated by disc diffusion assay against a panel of bacteria and fungi. A. littoralis leaf extract inhibited the growth of 8 of the 14 bacteria tested (57%). Gram-positive and Gram-negative bacteria were both inhibited by A. littoralis extract although Gram-positive bacteria were more susceptible. 5 of the 11 Gram-negative bacteria (45%) and 100% of the Gram-positive bacteria tested had their growth inhibited by A. littoralis extract. A. littoralis leaf extract displayed no antifungal activity towards any of the fungi when tested by disc diffusion assay. The antibacterial activity of A. littoralis leaf extract was further investigated by growth time course assays which showed significant growth inhibition in cultures of Bacillus
cereus, Bacillus subtilis and Pseudomonas fluorescens within 1 h but not of Aeromonas hydrophilia.

INTRODUCTION

Allocasuarina littoralis (black she-oak; previously known as Casuarina littoralis and Casuarina suberosa), a member of the genus Allocasuarina (family Casuarinaceae) are native trees to Australia, Southern Asia and the Pacific islands (Yasodha et al., 2004). Some members of the Casuarinaceae family (Casuarina cunninghamiana, Casuarina equisetifolia, Casuarina glauca) have also been introduced to other areas such as USA and the Caribbean (Staples et al., 2000) and Southern Africa (Henderson, 1995) where they are considered invasive species.

Members of the Casuarinaceae family were important as a food source to the Aboriginal people of Australia prior to European settlement. The Aborigines in the Canberra region are known to have eaten the leaves and young cones of Allocasuarina verticellara as a food (Nash, 2004). The Casuarinaceae family also had a role as traditional bush medicines for Australian Aborigines. The bark of Casuarina equisetifolia was used as an astringent and to treat diarrhoea, dysentery, headache and fever (Maiden, 1913; Maiden, 1889). On Groote Eylandt in the Northern Territory of Australia, the twigs were soaked in water and used as a mouthwash (Maiden, 1889). No evidence was found of Australian Aborigines, nor any other ethnic group, using Casuarinaceae as antimicrobial agents.

A recent report has demonstrated the antibacterial activity of a related Casuarinaceae species (Casuarina equisetifolia) against a variety of bacterial species (Parekh et al., 2005). Studies within this laboratory (Cock, 2008) have also found antibacterial activity in methanolic extracts of A. littoralis leaves against a limited panel of bacteria. The current study was undertaken to validate and extend these observations against a wider panel of bacteria and fungi.

MATERIALS AND METHODS

PLANT COLLECTION AND EXTRACTION

The extracts investigated in this study have been described previously (Cock, 2008). Briefly, Allocasuarina littoralis leaves were collected from Toohey Forest, Brisbane, Australia and were identified with reference to a taxonomic key to Toohey Forest plants (Coutts and Catterall, 1980). A. littoralis leaves were dried in a Sunbeam food dehydrator and the dried material was ground to a coarse powder. 1 g of the powdered leaves was extracted extensively in 50 ml methanol (Ajax, AR grade) for 24 hours at 4 ° C with gentle shaking. The extract was filtered through filter paper (Whatman No. 54) under vacuum followed by drying by rotary evaporation in an Eppendorf concentrator 5301. The resultant pellet was dissolved in 15 ml 20 % methanol. The extract was passed through 0.22 µm filter (Sarstedt) and stored at 4 ° C.

TEST MICROORGANISMS

All media was supplied by Oxoid Ltd. All microbial strains were obtained from Tarita Morais, Griffith University. Stock cultures of Aeromonas hydrophilia, Alcaligenes feacalis, Bacillus cereus, Bacillus subtilis, Citrobacter freundii, Enterobacter aerogenes, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeuroginosa, Pseudomonas fluorescens, Salmonella salford, Serratia marcescens, Staphylococcus aureus and Yersinia enterocolitia were subcultured and maintained in nutrient broth at 4 ° C. Aspergillus niger, Candida albicans, and Saccharomyces cerevisiae were maintained in Sabouraud media at 4 ° C.

EVALUATION OF ANTIMICROBIAL ACTIVITY

Antimicrobial activity of A. littoralis leaf extract was determined using a modified Kirby-Bauer (Bauer et al., 1966) disc diffusion method. Briefly, 100 µl of the test bacteria/fungi were grown in 10 ml of fresh media until they reached a count of approximately 10^{8} cells ml^{-1} for bacteria, or 10^{5} cells ml^{-1} for fungi. 100 µl of microbial suspension was spread onto agar plates corresponding to the broth in which they were maintained.

The extract was tested using 6 mm sterilised filter paper discs. Discs were impregnated with 10 µl of the test sample, allowed to dry and placed onto inoculated plates. The plates were allowed to stand at 4 ° C for 2 hours before incubation with the test microbial agents. Plates inoculated with A. feacalis, A. hydrophilia, B. cereus, B. subtilis, C. freundii, K. pneumoniae, P. aeuroginosa, P. fluorescens, S. marcescens, Y. enterocolitia, C. albicans and S. cerevisiae were incubated at 30 ° C for 24 hours, then the diameters of the inhibition zones were measured in millimetres. Plates inoculated with E. aerogenes, E. coli, S. Salford and S. aureus were incubated at 37 ° C for 24 hours, then the diameters of the inhibition zones were measured. A. niger inoculated plates were incubated at 25 ° C for 48 hours then the zones of inhibition were measured. All measurements were to the closest whole millimetre. Each antimicrobial assay was performed in at least triplicate. Mean values are reported in this report. Standard discs of ampicillin (2 µg), chloramphenicol (10 µg) or ciprafloxicin (2.5 µg) were obtained from Oxoid Ltd. and served as positive controls for antimicrobial activity. For fungi, nystatin discs (100 µg, Oxoid Ltd.) were used as a positive control. Filter discs impregnated with 10 µl of distilled water were used as a negative control.

BACTERIAL GROWTH TIME COURSE ASSAY

3 ml of bacterial cultures (B. cereus, B. subtilis, A. hydrophilia, P. fluorescens) in nutrient broth were 27 ml nutrient broth containing 0.5 ml A. littoralis extract (diluted 1 in 10 in sterile deionised water). The tubes were incubated at 30 ° C with gentle shaking. The optical density was measured at 550 nm after 0, 1, 2, 4 and 6 h incubations. Control tubes were incubated under the same conditions but without the extract. All assays were performed in triplicate.

RESULTS AND DISCUSSION

A. littoralis leaf extract was diluted to a 25 mg/ml concentration. 10 µl of the extract was tested in the disc diffusion assay against 17 microorganisms (table 1). The A. littoralis leaf extract inhibited the growth of 8 of the 14 bacteria tested (57%). The antibacterial activity was relatively strong against A. faecalis, P.fluorescens, S. Salford and S. aureus (as determined by the diameter of the zone of inhibition).

Figure 1

Table 1: Antibacterial activity of extract. Numbers indicate the mean diameters of inhibition of triplicate experiments Â± standard deviation. Â– indicates no growth inhibition. Chl indicates chloramphenicol (10 Âµg) was used as the positive control. Amp indicates ampicillin (2 Âµg) was used as the positive control. Cip indicates ciprafloxicin (2.5 Âµg) was used as the positive control. . Nys indicates nystatin nystatin discs (100 Âµg) was used as the positive control.

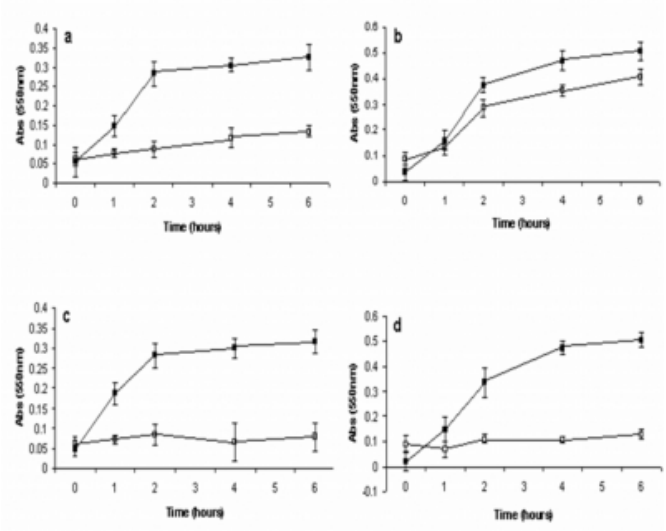
Microbial Species	Mean Zone of Inhibition ± SD (mm)	
	Antibiotic	*A. littoralis* extract
Gram negative rods		
Aeromonas hydrophilia	17.3 ± 0.6 (Chl)	-
Alcaligenes faecalis	13.3 ± 0.6 (Amp)	16.7 ± 1.2
Citrobacter fruendii	23.0 ± 1.0 (Chl)	-
Enterobacter aerogenes	17.3 ± 0.3 (Chl)	-
Escherichia coli	16.7 ± 0.6 (Amp)	-
Klebsiella pneumoniae	18.3 ± 0.6 (Amp)	10.0 ± 1.0
Pseudomonas aeuroginosa	31.6 ± 0.3 (Cip)	-
Pseudomonas fluorescens	21.0 ± 0 (Chl)	18.0 ± 0
Salmonella salford	25.3 ± 0.3 (Amp)	14.0 ± 0
Seratia marescens	25.7 ± 0.6 (Chl)	-
Yersinia enterocolitia	16.3 ± 0.3 (Amp)	10.0 ± 1.0
Gram positive rods		
Bacillus cereus	25.3 ± 0.6 (Chl)	9.6 ± 0.3
Bacillus subtilis	22.7 ± 0.6 (Amp)	9.3 ± 0.3
Gram positive cocci		
Staphylococcus aureus	16.3 ± 0.3 (Amp)	16.6 ± 0.3
Fungi		
Aspergillus niger	18.0 ± 0 (Cip)	-
Candida albicans	25.7 ± 0.6 (Nys)	-
Yeast		
Saccharomyces cerevisiae	21.3 ± 0.6 (Nys)	-

Both Gram-positive and Gram-negative bacterial growth were inhibited by A. littoralis leaf extract although a greater susceptibility of Gram-positive bacteria was apparent. Of the 11 Gram-negative bacteria tested, 5 (45%) were inhibited by A. littoralis extract. The extract inhibited the growth of all of Gram-positive bacteria tested. The greater susceptibility of Gram-positive bacteria has been previously reported for South American (Paz et al., 1995), African (Kudi et al., 1999; Vlietinck et al., 1995) and Australian (Palombo and Semple, 2001) plant extracts. Susceptibility differences between Gram-positive and Gram-negative bacteria may be due to cell wall structural differences between these classes of bacteria. The Gram-negative bacterial cell wall outer membrane appears to act as a barrier to many substances including antibiotics (Tortora et al., 2001). No antifungal activity was seen in the present study, although a limited panel of fungi was tested.

The antibacterial activity of the A. littoralis leaf extract was further investigated by bacterial growth time course assays in the presence and absence of the extract. The concentration of the extract used in these assays was 41.7 µg/ml. A. littoralis leaf extract was able to significantly inhibit B. cereus (figure 1a), P. fluorescens (figure 1c) and B. subtilis (figure 1d) growth within 1 h indicating a rapid antimicrobial action. Aeromonas hydrophilia (figure 1b) growth was not inhibited greatly by A. littoralis leaf extract, in agreement with previously reported results (Cock, 2008).

Figure 2

Figure 1: Inhibition of bacterial growth by methanolic extract of leaves against (a) , (b) , (c) , (d) . For all graphs, ? represent measured bacterial growth values for test cultures (with extract) and ? represent control bacterial growth values (no extract). Values are the mean of triplicate determinations.



In summary, these studies and previous studies within this laboratory (Cock, 2008) are the first studies that have demonstrated the antibacterial activity of A. littoralis leaf extracts. Both Gram-positive and Gram-negative bacteria were susceptible to A. littoralis leaf extract. The broad range of microbial susceptibilities indicates the potential of A. littoralis leaf extract as a surface disinfectant as well as for medicinal purposes and possibly as food additives to inhibit spoilage. However, further studies are needed before these extracts can be applied to these purposes. In particular, toxicity studies are needed to determine the suitability of these extracts for the use as antiseptic agents and as a food additive.

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